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Adaptive Significance of a Small Heat Shock/α-Crystallin Protein (p26) in Encysted Embryos of the Brine Shrimp, *Artemia franciscana*[1]

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SYNOPSIS. **Encysted embryos of the primitive crustacean, *Artemia franciscana*, are among the most resistant of all animal life history stages to extremes of environmental stress. This resistance has likely been acquired during evolutionary adaptation to their harsh ecological setting. In the laboratory, for example, some of these embryos survive more than 6 years of continuous anoxia while fully hydrated and at room temperature, a phenomenal capability that can easily be related to their frequent encounters with anoxic conditions in nature. A small heat shock protein belonging to the α-crystallin family of proteins, referred to as p26, appears to play a central role in the stress resistance of these embryos. Our evidence shows that this protein is an important component in their adaptive repertoire, and vital for the survival of *Artemia* in nature.**

INTRODUCTION

In the context of resistence to environmental stress, the brine shrimp *Artemia* qualifies as a "Krogh animal," based on the famous physiologist August Krogh's statement that "For a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied" (Krogh, 1929). This primitive anostracan crustacean is found in a wide variety of very harsh, usually hypersaline environments world-wide, the more than 500 described locations ranging from sea level to 4,500 m, with widely fluctuating temperatures (5–40°C) and salinities (75–240 ppt) (Triantaphyllidis *et al.*, 1998; Van Stappen *et al.*, 1998). Thus, it is not surprising that the active (motile) life history stages of this animal exhibit extraordinary osmoregulatory abilities and, considering the temperature range of their environments, impressive resistance to thermal fluctuations. These abilities are based on a variety of adaptations encompassing all levels of biological organization (see books by Persoone *et al.*, 1980; Decleir *et al.*, 1987; Warner *et al.*, 1989; MacRae *et al.*, 1989; Browne *et al.*, 1991). Even more remarkable is the stress tolerance of the encysted embryos (cysts) that *Artemia* can produce, and these are the focus of this paper. Particular attention will be given to an abundant small heat shock/α-crystallin protein (p26) and its participation in the adaptive repertoire of these embryos, but the compatible solute trehalose and the heat shock protein families Hsp 70 and 90 will also be considered.

In beginning this paper we note the observation of Feder and Hofmann (1999) that "The relevant literature on Hsps and molecular chaperones is huge, now encompassing more than 12,000 references. Even a review of the relevant reviews is difficult." Thus, no attempt will be made to consider that literature in any detail; the excellent review of Feder and Hofmann (1999) is recommended. Those authors point out that the significance of heat shock proteins and molecular chaperones in the areas of physiological ecology and evolution has yet to be realized, and certainly worthy of further study (also see Feder and Block, 1991; Coleman *et al.*, 1995), views with which we agree.

LIFE HISTORY OF *ARTEMIA FRANCISCANA*

Of the described species of the genus *Artemia* (Triantapyllidis *et al.*, 1998) most research has focused on *A. franciscana* whose life history is outlined in Figure 1. This spe-

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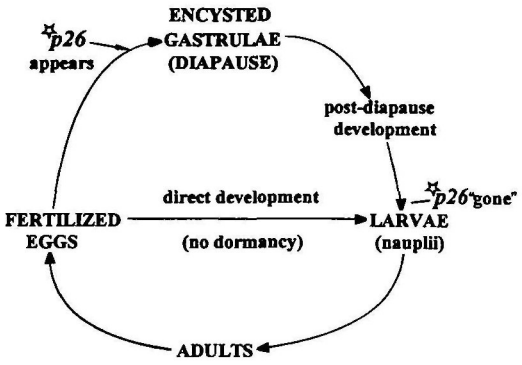

FIG. 1. Simplified diagram of the life cycle of *Artemia franciscana.*

cies is bisexual, with adults producing zygotes that proceed along one of two developmental paths, depending on conditions (Drinkwater and Crowe, 1987; Drinkwater and Clegg, 1991; Van Stappen *et al.*, 1998). The path of direct development (Fig. 1) results in the release of fully-formed, swimming larvae. The alternative is the production of encysted gastrula embryos that enter diapause (an obligate state of developmental arrest) and are then released into the usually hypersaline environment. These embryos remain in diapause until experiencing conditions that terminate it, and the resumption of development then ensues, given permissive conditions of temperature, water content and oxygen (Clegg and Conte, 1980; Lavens and Sorgeloos, 1987; Drinkwater and Clegg, 1991; Van Stappen *et al.*, 1998). Of some importance is the synthesis of p26 during the production of diapause embryos, an event that does not occur during the direct pathway of development, or in any other stage of the life cycle (Fig. 1). Much more will be said about this protein, but first we consider the encysted embryo in more detail since the adaptive significance of p26 must be evaluated in that context.

The Encysted Embryo

Figure 2 describes the general ultrastructure of an encysted gastrula. These embryos are arguably among the most resistant of all animal life history stages to environmental extremes that range from complete desiccation, to intense UV radiation to years of continuous anoxia (see Clegg and Conte,

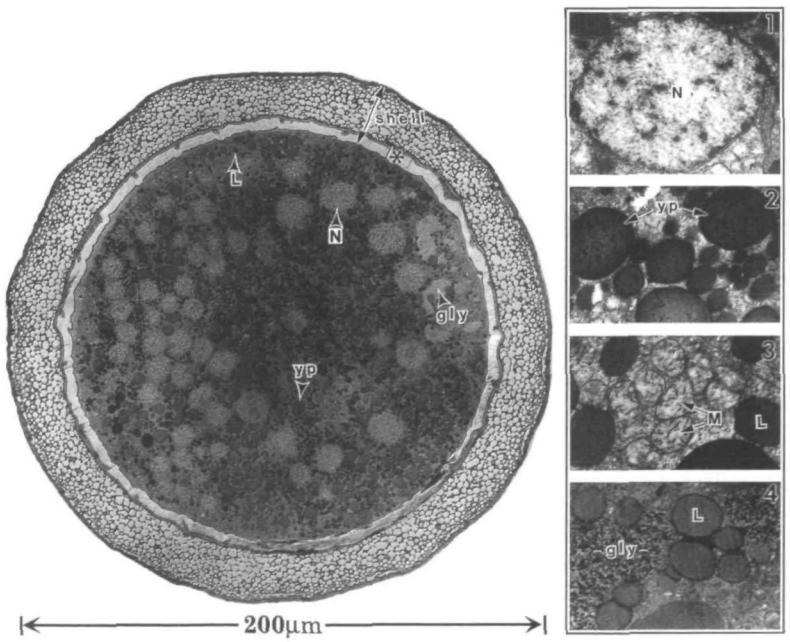

FIG. 2. Ultrastructure of a post-diapause (activated) encysted embryo of *A. franciscana.* The panels to the right illustrate major organelles at higher magnification. L (lipid droplets), N (nuclei), yp (yolk platelets), gly (glycogen), M (mitochondria), asterisk (inner embryonic cuticle).

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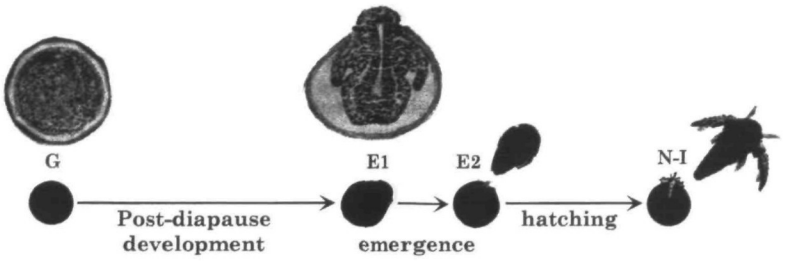

FIG. 3. Development of an activated (post-diapause) encysted gastrula (G). Emerged embryos (E1, E2) give rise to the swimming nauplius larva (N-I).

1980; Lavens and Sorgeloos, 1987; Hand,1997, 1998; Clegg and Jackson, 1998). Encysted embryos normally encounter these conditions in nature when floating on the surface of their hypersaline environment and/or after being blown on shore where they frequently form "wind rows" and are buried under masses of decaying biological material. In the latter case, the embryos experience severely hypoxic or anoxic conditions, as well as unpredictable and repeated bouts of desiccation/hydration due to fluctuations in humidity or rain. Thus, the diapause embryo must be equipped to face a barrage of stresses that commonly take place long after they are released from females. Figure 2 and other ultrastructural observations (Hofmann and Hand, 1990) indicate that the encysted gastrula is a typical yolky crustacean embryo.

One of the conditions leading to the termination of embryonic diapause in some populations of *A. franciscana* is desiccation (Drinkwater and Clegg, 1991). In this case the embryos are "activated" in the sense that diapause has ended, but they are "quiescent" since their development is prevented due to lack of water. When sufficient water is present, the embryos resume development, the only other requirements being suitable temperatures and adequate levels of molecular oxygen (Fig. 3). Post-diapause development is characterized by the absence of DNA synthesis and cell division, neither of which resume until emergence of the E_1 stage (Nakanishi *et al.*, 1962, 1963; Olson and Clegg, 1978; Clegg and Conte, 1980). This unusual morphogenesis can also be interpreted as a significant adaptation because the embryo retains its resistant capabilities during post-diapause development, losing them only after the embryos emerge from their surrounding shells (Warner *et al.*, 1997). Thus, the occurrence of DNA synthesis and cell division could be a real problem for embryos that must suddenly, and without warning, undergo anoxia, thermal challenge or desiccation (or even all three). It is also significant that the level of p26 remains at a high level during post-diapause development, not being reduced to low levels until emergence occurs (E1 in Fig. 3) (Clegg *et al.*, 1994). After the first stage nauplius molts, only a trace of p26 remains, and this is restricted to a few cells that do not divide and eventually die (Liang and MacRae, 1999), a significant point to which we will return.

PROLONGED ANOXIA IN ACTIVATED ENCYSTED EMBRYOS

In general, animals that are well-adapted to experiencing anoxic conditions in nature rarely survive the continuous absence of molecular oxygen for more than a month in the laboratory (Storey and Storey, 1990; Bryant, 1991; Hochachka *et al.*, 1993; Grieshaber *et al.*,1994; Guppy *et al.*, 1994; Hand and Hardewig, 1996). In that regard, it is nothing short of spectacular that the activated, fully hydrated encysted embryos of *Artemia* survive anoxia at temperatures of 21–23°C for periods of *years*. Figure 4 shows that almost 60% give rise to normal larvae after experiencing 4 years of continuous anoxia (data from Clegg, 1994, 1997), about 35% survive 5 years under these conditions (data from Clegg and Jackson, 1998) and even after more than 6 years of anoxia almost 10% hatch when returned to

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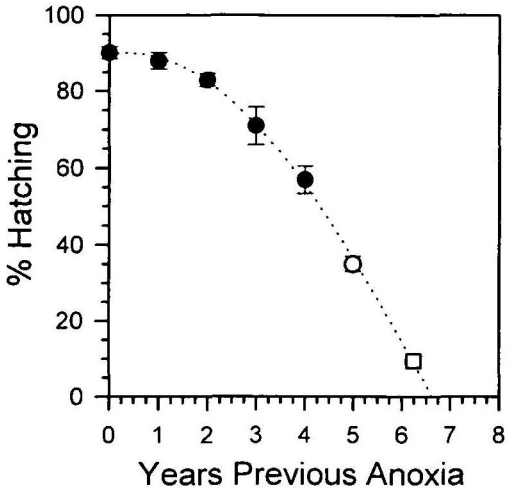

FIG. 4. Percentage of encysted embryos producing normal swimming nauplii (hatching) after continuous anoxia for the times shown. Hatching was in aerobic seawater and observations were continued until hatching was complete. Solid circles are data from Clegg (1994, 1997) and the open circle is from Clegg and Jackson (1998). The open square represents data not previously published. Means ± standard errors are shown (or are within the symbols); number of observations have been given in the publications noted above, except for the 6.3 year measurements where 3 groups of at least 200 embryos were used. The dotted line is drawn from a third-order regression ($r^2 = 0.999$) and predicts that hatching would be reduced to zero after 6 years and 7.5 months of anoxia.

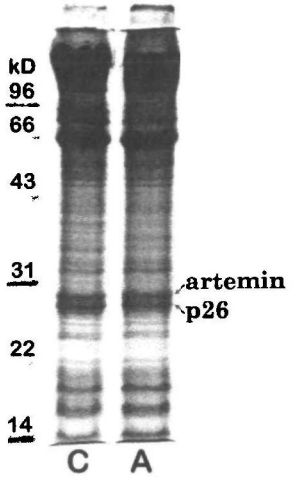

FIG. 5. SDS-PAGE of whole homogenates of control encysted embryos (C) and from embryos previously undergoing 6.3 years of anoxia (A). The latter were from the same sample of cysts represented by the open square in Figure 4. Molecular weight markers and other proteins were detected by Coomassie staining. Two specific proteins are indicated by the arrows.

aerobic conditions (data not previously published). A case has been made (Clegg, 1997) that the metabolism of anoxic embryos must be brought to a reversible standstill to explain these and other results, including microcalorimetric studies (see Hontoria *et al.*, 1993; Hand, 1999) and a variety of measurements on embryo metabolites (Clegg, 1997; Clegg *et al.*, 1996; Clegg and Jackson, 1998). Such a conclusion requires that the anoxic embryos maintain their integrity in the apparent absence of biosynthesis and ongoing energy metabolism. In the present case we consider particularly the question of protein stability in anoxic embryos.

One threat to protein integrity in anoxic embryos is proteolysis in the absence of biosynthesis. Work by Anchordoguy and Hand (1994 and 1995) revealed substantial inhibition of the ubiquitin pathway of proteolysis in anoxic embryos, while other evidence indicated that anoxia reduced proteolytic activity of all kinds to a standstill (Clegg, 1997; Warner *et al.*, 1997; Hand, 1997 and 1998). Further indication of the latter is shown in Figure 5 which compares proteins from embryos that had previously undergone over 6 years of anoxia (Fig. 4, open square) with those from controls. The protein profiles of these two samples are remarkably similar, including p26, indicating that little if any proteolysis had taken place during 6 years of anoxia, even though about 90% of these embryos are dead (that is, they do not hatch under permissive conditions). It seems clear that proteolysis is under remarkably tight control in these embryos.

Equally serious is the potential problem of protein unfolding and aggregation in long-term anoxic embryos (see Clegg, 1997). Hydrated globular proteins can be expected to be unstable (Dill, 1990; Creighton, 1990; Shoichet *et al.*, 1995) in the sense that they must be flexible to function at *physiological temperatures* (Somero, 1995) and to be flexible is to be unstable. Thus, it seemed unlikely that these problems could be avoided through the evolution of uniquely stable proteins. Rather,

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some other way to maintain protein integrity was needed and, as we take up in the next section, p26 seems to play a key role in this regard through its ability to act as a molecular chaperone. Before doing that we should point out another protein in these embryos (Fig. 5) that is as abundant as p26, called "artemin" purified and characterized by De Graaf *et al.*, (1990) who also review the earlier literature. Its function is not known, but it shows slight sequence similarity to ferritin. Furthermore, the synthesis of artemin is upregulated strongly during aerobic recovery after extended anoxia behaving, in this regard, like a stress protein (Clegg and Jackson, 1998). Artemin deserves further study.

p26, A Small Heat Shock/α-Crystallin Protein

In this section we summarize a number of features of this protein obtained in previous studies (Clegg *et al.*, 1994, 1995; Jackson and Clegg, 1996; Liang *et al.*, 1997*a, b,* Liang and MacRae, 1999). These features have been discussed in detail in those papers, and that will not be repeated here; however, some coverage is needed for background and to evaluate the adaptive significance of p26.

1. The native protein has a molecular mass of about 700kD (27 sub-units, each about 26kD) and appears as a particle of about 15 nm in diameter under the electron microscope.
2. It is present only in the encysted embryo where it accumulates to a massive 10–15% of the total non-yolk protein, to our knowledge the highest level of any constituitive stress protein.
3. The synthesis of p26 has not been induced by stress under any of various conditions examined, but instead is programmed only in embryos destined to enter diapause. Thus, the accumulation of this protein, in a sense, pre-meditates stresses that the embryos may experience years or even decades later.
4. When activated encysted embryos are exposed to anoxia or thermal shock, about half of the p26 in the cytoplasm is translocated into the nucleus, and this is reversed when the stress is removed.
5. Indirect evidence suggests that p26 also interacts with a wide variety of cytoplasmic proteins in anoxic embryos, and these interactions are reversed when aerobic conditions are restored.
6. Nuclear/cytoplasmic translocations of p26 carried out *in vitro* show strong pH dependence: acidic pH favors translocation into nuclei, and that is reversed by alkaline pH. The pH-dependence of p26 translocations *in vitro* can be related very nicely to what happens in intact embryos: aerobic embryos have an intracellular pH $\geq$ 7.9, whereas anoxia reduces this to about 6.6 (Busa *et al.*, 1982; Busa and Crowe, 1983; recently reviewed by Hand, 1997, 1998, 1999).
7. p26 exhibits impressive molecular chaperone activity *in vitro,* rescueing previously heat inactivated citrate synthase and preventing freeze-thaw induced aggregation of that enzyme.
8. This protein has been completely sequenced and found to bear strong resemblence to the small heat shock/α-crystallin protein superfamily (de Jong *et al.*, 1998). One of several interesting features is the absence of a typical nuclear localization signal, in spite of its extensive translocations between cytoplasm and nucleus, a puzzling observation.

Finally, we note that Liang and MacRae (1999) have shown that *Escherichia coli* transfected with the gene for p26 and expressing this protein are much more thermotolerant than are bacteria containing only the plasmid. Those results support the substantial amount of *in vitro* data and provide evidence that p26 does indeed function as a molecular chaperone in the cells of the intact encysted embryo.

Is p26 Restricted to Artemia?

A wide variety of invertebrates have been examined for p26, including the resting stages of other crustaceans (our unpublished results). Thus, no p26 was detected by Western blotting in cysts of the fairy shrimp, *Branchinecta sandiegonensis* a

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closely related, sister species of *Artemia* (see Spears and Abele, 1999), cysts of the notostracan *Triops sp.* or ephippia of the cladoceran, *Daphnia sp.* Furthermore, Coomassie staining failed to reveal significant amounts of a protein anywhere near 26kD in all three samples. A caveat in these studies is the poor cross-reactivity of antibodies against small heat shock proteins (see Fiege *et al.*, 1996). Nevertheless, at this point there is no evidence that p26 exists outside the genus *Artemia.*

Encysted embryos from all of the following *Artemia* species contain p26 (and artemin) in amounts comparable to *A. franciscana* from the San Francisco Bay area (unpublished results): *parthenogenetica* (Siberia and France); *sinica* (China); *urmiana* (Iran); *salina* (Tunisia); *monica* (USA). We suspect that these proteins are present in all brine shrimp species throughout the world. If so, natural selection has retained p26 and artemin in spite of the vastly different ecological settings of many of these species, some of which have probably been genetically isolated for many millions of years (see Browne and Bowen, 1991). Interestingly, encysted embryos of *A. monica* do not undergo desiccation in nature and are not very good at surviving this treatment in the laboratory (Drinkwater and Crowe, 1987); nevertheless, they contain p26 in amounts equivalent to those in *A. franciscana* embryos. This leads us to speculate that p26 might not play a major role in desiccation tolerance *per se,* that function being performed primarily by the compatible solutes present in abundance in these embryos.

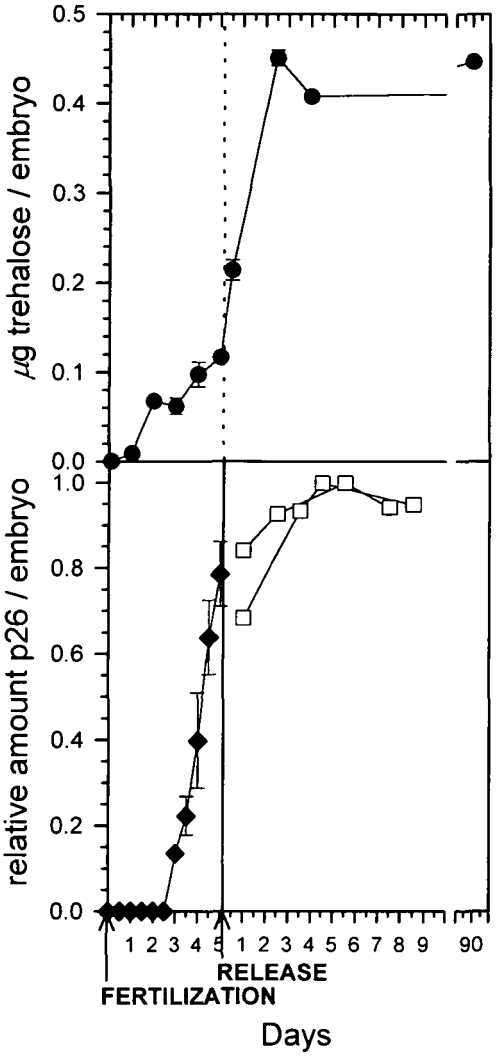

FIG. 6. Contents of trehalose and p26 per encysted embryo as a function of time after fertilization. The vertical lines in each case represent the time of release of encysted embryos from females. Data for trehalose are re-drawn from Clegg and Jackson (1998) and the solid triangles are p26 data taken from Jackson and Clegg (1996). Open squares show previously unpublished data.

RELATIONSHIP BETWEEN P26 AND TREHALOSE ACCUMULATION DURING FORMATION OF DIAPAUSE EMBRYOS

Although this paper focuses chiefly on the stress protein p26, other factors contribute to the phenomenal stability of encysted embryos. Prominent among these is the disaccharide trehalose, a compatible solute that accumulates to about 15% of the embryo's dry mass (see Clegg and Conte, 1980). This sugar is a well-known stabilizer of macromolecules and membranes (see Crowe *et al.*, 1992, 1998; Welch and Brown, 1996) so its appearance during the development of these embryos merited study. In Figure 6 we show previously published data (Clegg and Jackson, 1998) on the developmental accumulation of trehalose in embryos destined for diapause (top, Fig. 6). Noteworthy is the fact that most of the trehalose is made after diapause embry-

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os are released. Therefore, *developmental* diapause takes place prior to the reduction in overall metabolic rate which requires about a week or so after release to reduce it to unmeasurable levels (Clegg *et al.*, 1996; Clegg and Jackson, 1998). The lower part of Figure 6 describes the corresponding pattern for p26 accumulation. The solid symbols are from Jackson and Clegg (1996) while the post-release pattern is shown by open symbols (previously unpublished data). Although the pre- and post-release data were obtained using separate Western blots the result suggests that, unlike trehalose, most of the p26 present in these embryos is produced prior to their release from females. Interestingly, a number of studies have been carried out on interactions between trehalose and stress proteins in yeast (for this literature see Singer and Lindquist, 1998). For example, Singer and Lindquist (1998) found that trehalose did indeed stabilize proteins during heat shock (and, importantly, suppressed protein aggregation) but that this sugar also interfered with chaperone-mediated protein refolding following bouts of heat stress. Singer and Lindquist (1998) also found that trehalose was rapidly removed following heat shock, presumably to allow chaperone function. In the *Artemia* encysted embryo the situation must be different, at least with regard to p26, since trehalose is actually synthesized and maintained at high levels following heat stress (Clegg and Jackson, 1992). It will be interesting to evaluate the influence of trehalose on the performance of p26 as a molecular chaperone *in vitro*.

Attempts to Induce Thermotolerance in Encysted Embryos

In previous sections we have taken the position that p26 is not synthesized in response to environmental stress, per se, but rather in pre-mediation of stresses to be encountered long after the diapause embryo is formed. Thus, the possibility arises that diapause embryos are maximally prepared for encounters with stress. Therefore, we asked whether it was possible to further enhance stress tolerance in the encysted embryo. We attempted to induce thermotolerance in activated encysted embryos by means of the traditional approach of heat-shocking embryos and evaluating their performance when later exposed to an otherwise lethal heat treatment (Fig. 7). Dried embryos were first hydrated at 2–4°C for 16–18 hr and then either assayed directly for p26 and hatching (controls) or given various thermal exposures: sublethal heat shock (SHS) was 3 hr at 42°C, lethal heat treatment (LHT) was 2 hr at 50°C and the attempt to induce thermal tolerance (ITT) was evaluated, in this case, by a LHT immediately after SHS. In all the foregoing studies the embryos were heated from 22°C to the desired temperature at 0.7°C/min to avoid transfer directly from 2–4°C. Hatching assays were carried out in aerobic seawater as described previously (Clegg, 1997). The results in Figure 7 show clearly that no induction of thermal tolerance took place under these conditions. Other temperature-time regimes have been examined, as well as allowing a recovery period of 2 and 4 hr after SHS before applying the lethal heat treatments; however, in no case have we been able to demonstrate enhanced thermotolerance. These results are in agreement with the statement of Miller and McLennon (1988*a*) that thermotolerance could not be induced in encysted embryos, although they showed that thermotolerance could easily be induced in larvae hatched from them. Liang and MacRae (1999) also explored this matter, employing several different time-tem-

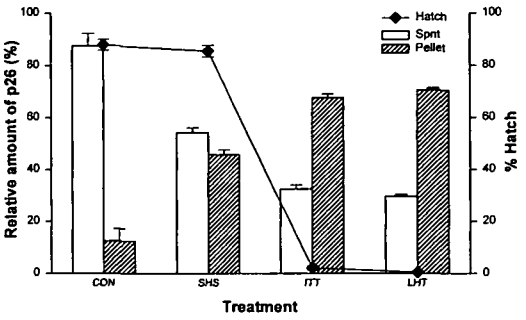

FIG. 7. p26 location and attempts to induce thermotolerance in encysted embryos. CON = controls; SHS = sublethal heat shock; ITT = lethal heat treatment after previous SHS; LHT = lethal heat treatment alone. See text for details. p26 was quantified by Western blotting. Spnt and Pellet refer to fractions of homogenates obtained by centrifugation at 1630xg for 5 minutes at 2°C (see Clegg *et al.*, 1994 for details).

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perature protocols, with and without a recovery period between SHS and LHT, but they also could not induce thermotolerance in encysted embryos.

Figure 7 also shows the effects of various thermal conditions on the nuclear translocation of p26, done conveniently by measuring the amount of this protein in low speed (1630xg, 5 minutes) pelleted fractions that we have shown reliably reflects the distribution of p26 between nucleus and cytoplasm (see Clegg *et al.*, 1994 and 1995; Liang *et al.*, 1997*a, b*). Those studies describe in detail the procedures used to detect p26 by Western immunoblotting. As expected from previous work (Liang *et al.*, 1997*a*), a small amount of p26 was associated with the nuclear fraction of control embryos, and Figure 7 shows that this amount was increased substantially by SHS. Although a direct LHT, or one given after SHS (ITT) significantly increased the amount of p26 in nuclei, this translocation had no benefit in terms of embryo survival (Fig. 7).

The results in Figure 7, along with those of Miller and McLennan (1988*a, b*) and Liang and MacRae (1999) support the conjecture that these embryos are as stress-resistant as possible. In view of that interesting possibility, and because it seemed appropriate to evaluate the effects of heat shock on the behavior of stress proteins other than p26, we examined two likely candidates, the Hsp 70 and 90 families (Morimoto *et al.*, 1994; Fiege *et al.*, 1996; Hartl, 1996: Fink and Golo, 1998)

Effects of Heat Shock of the Relative Amounts and Distributions of the HSP 70 and 90 Families in Encysted Embryos

Activated embryos were pre-hydrated as described above and then either assayed at once for these stress proteins by Western blotting (C, control, Figure 8) or taken from 22°C to 50°C (0.7°C/min) and kept there for up to 2 hr prior to assay. Although substantial p26 translocation into nuclei took place, as expected, no such effects were apparent for the other two heat shock protein families (Fig. 8). This is interesting and rather surprising since both Hsp 70 and 90 have been shown to undergo substantial translocation into the nuclei of a wide variety of cells and tissues under heat shock conditions; in fact, there have been so many such studies that we cite only a few recent reviews for Hsp 70 (Karlin and Brocchieri, 1998; Kiang and Tsokos, 1998) and Hsp 90 (Csermely *et al.*, 1998; Yahara, 1998). Of course, the data in Figure 8 by no means rule out the participation of hsp-70 and 90 in the overall stress response, only that they do not undergo the expected nuclear translocation. These results, taken with the failure of three laboratories to induce thermal tolerance (Fig. 7) support the hypothesis that encysted embryos have "perfected" their ability to successfully encounter a variety of environmental hazards long after they are formed and enter diapause, without the need for synthesis of additional stress proteins. It appears to us that the accumulation in the diapause embryo of massive amounts of the molecular chaperone/stress protein, p26, plays a major role in this adaptation.

Concluding Remarks

We began this paper by suggesting that *Artemia* fits the principle that August Krogh proposed 70 years ago; thus, if one is interested in relating cellular and molecular adaptations associated with stress resistance to the natural ecological settings of organisms, the encysted embryos of this animal clearly provide special opportunities for study and, in some respects, unique ones. Trehalose and p26 are obviously not the only adaptations associated with stress resistence in these remarkable embryos. For instance, the surrounding shell provides not only physical and mechanical protection, but the impermeability of its inner layer to non-volatile solutes allows the embryos to avoid being swamped by inorganic ions from their hypersaline environment, as well as preventing the loss of embryonic metabolites and other constituents. More study is required to understand all the ingredients that make up the "adaptive repertoire" of encysted embryos, but it seems certain now that p26 and trehalose must be involved in a major way.

Is the role of p26 limited to chaperoning

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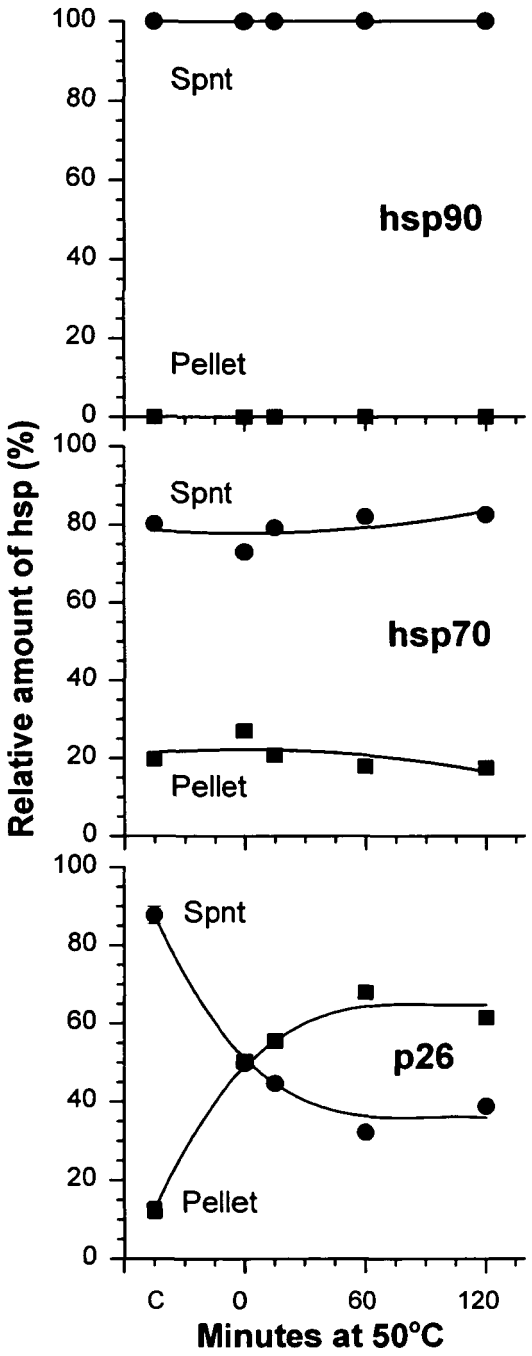

FIG. 8. Heat shock proteins in low speed fractions of encysted embryos (see Figure 8 legend) exposed to 50°C for the times shown. Controls (C) were not exposed to heat treatment; others were brought to 50°C from 22° by heating at 0.7°C/min, and then held at 50°C for the times shown. Relative amounts of these proteins were estimated by conventional Western blotting, using antibodies against the hsp90 family

proteins and, probably other macromolecules? Previously in this paper we pointed out the interesting correlation between the appearance of p26 in the diapause-destined embryo (Fig. 6, bottom) and the cessation of DNA synthesis and cell division at about this same point in development (Nakanishi *et al.*, 1962, 1963; Olson and Clegg, 1978; Clegg and Conte, 1980). Similarly, the substantial decrease in the level of p26 at and after emergence (E1, E2 in Fig. 3) is closely correlated with the resumption of DNA synthesis and cell division. These correlations, plus the inability to induce p26 in all other life history stages in spite of repeated attempts, allows for the interesting possibility that p26 might be involved in the inhibition of DNA synthesis and cell division, either as part of the protection of DNA and other nuclear constituents, or in a manner unrelated to such protection.

The ability of at least some encysted *Artemia* embryos to survive more than 6 years of continuous anoxia (Fig. 4) has significance that transcends stress resistance. The case has been made (Clegg, 1997) that anoxia brings embryonic metabolism to a reversible standstill. However, we note here, and elsewhere (Clegg, 1997) that the requirement for a more or less constant flow of free energy to maintain cellular (and organismic) integrity is a *major* axiom of biology. Thus, available evidence indicates that anoxic encysted embryos may provide an extraordinary exception to this central concept and, as a result, could be of utility in achieving a better understanding of the relationship between biological (cellular) stability and energy metabolism. Does the unusual independence of the anoxic encysted embryo from the requirement for free energy flow represent simply "an exception that proves the rule?" Srere (1997) and Jenkins (1997) point out that this much used phrase is usually used incorrectly. As

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(StressGen, Canada, SPA-840), the hsp70 family (Affinity Bioreagents, USA, MA3-001) and our own affinity-purified anti-p26 (Clegg *et al.*, 1994). A single isoform of hsp90 was detected; the two hsp70 isoforms present (68 and 72 kD) were summed to produce the data in this figure.

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Rall (1994) writes, "prove" in that phrase derives from the Latin *probere,* which generally means "to test," and he concludes: "Hence, an exception that proves the rule in fact tests the rule and if exceptions are found the rule is false." Are encysted embryos of *Artemia* the only such "exception"? Probably not (Clegg and Jackson, 1998), in view of the astonishing longevity of hydrated copepod embryos recovered from anoxic sediments beneath fresh water ponds (Hairston *et al.*, 1995) the Baltic Sea (Katajisto, 1996) and a Rhode Island estuary (Marcus *et al.*, 1994). Also worth noting is the study by Reiswig and Miller (1998) showing that the stress-resistant gemmules of a freshwater sponge survive at least 4 months of continuous anoxia while hydrated at about 20°C with no decline in viability. Those authors concluded, tentatively, that such results could be explained by the anoxia-induced cessation of gemmule metabolism. We suspect that this use of a reversible cessation of metabolism, or "cryptobiosis" (Keilin, 1959) while hydrated under anoxic conditions is not restricted to these crustaceans. Since *Artemia* embryos have acquired this ability during their evolution, there seems to be no reason why its occurrence in other organisms should not be possible.

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